Two mRNA Transcripts (rBAT-1 and rBAT-2) Are Involved in System b(+)P-related Amino Acid Transport*

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Previously, we isolated a cDNA clone (rBAT-1) of 2.2 kilobase pairs (kb) from a rabbit kidney cortex cDNA library, encoding a protein involved in sodium-independent transport of L-dibasic amino acids, L-cystine, and some neutral amino acids via a system related to b(+)P-like activity (Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M., and Murer, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5601–5605). In Northern blot hybridization using an rBAT-1 cDNA probe, 2.2- and 3.9-kb mRNA species were observed. Here we describe the isolation of a 3.9-kb cDNA clone (rBAT-2) by expression cloning using Xenopus laevis oocytes corresponding to the 3.9-kb mRNA species. On the basis of sequence analysis, in vitro translation (major protein of approximately 78 kDa), and functional analysis (expression of transport function), we conclude that rBAT-1- and rBAT-2-related proteins are identical: 677 amino acids in length, with most likely only one transmembrane-spanning domain. There are seven differences in the nucleotide composition within a common overlap of 2189 nucleotides, resulting in 2 amino acid replacements. In comparison with rBAT-1, rBAT-2 has 26 additional nucleotides at the 5'-end, an identical location of the first polyadenylation signal, and approximately 1.7 kb of 3'-untranslated sequence (rich in AT(U) motifs) prior to a poly(A) tail of 63 adenines. We conclude that rBAT-1 and rBAT-2 encode the same protein and that the major difference seems to be related to the use of different polyadenylation signals.

Amino acids are transported across mammalian plasma membranes by multiple sodium-dependent and -independent pathways. Classification into distinct transport pathways is mainly based on studies related to substrate specificity, including cross-inhibition studies, as well as on studies characterizing the involvement of a sodium gradient as a driving force (1-4). It seems that two different sodium-independent transport pathways are involved in transmembrane transport of dibasic amino acids: (i) system y*, which accepts L-dibasic amino acids and excludes L-cystine (it interacts with some neutral amino acids (e.g. L-homoserine), but only in the presence of sodium (1, 2, 5-7); (ii) system b(+)P, which accepts L-dibasic amino acids, L-cystine, and some neutral L-amino acids (e.g. L-leucine and L-alanine). In contrast to system y*, system b(+)P interacts with L-homoserine in the presence and absence of sodium (1, 2, 8-10).

Little is known about the structural identity of the different amino acid transporters. Only recently, evidence was obtained that a murine ecotropic retrovirus receptor most likely represents a protein closely related to system y* (5-7). In addition, functional cloning using the Xenopus laevis oocyte expression system has led to the identification of a rat kidney protein and a rabbit kidney protein, which have high potencies to induce transport activities in X. laevis oocytes resembling system b(+)P-like transport activity (8-13). Although the rat kidney protein identified by Tate and colleagues (11) was closely related ("identical") to that cloned from the same source by Wells and Hediger (12), the former authors suggested the participation of the protein in amino acid transport via a system L-related pathway (11, 12). The rat kidney protein identified by Wells and Hediger was named D2 (12), and the corresponding protein identified from rabbit kidney was named rBAT (rBAT-1 in the present paper); it was concluded that rBAT (rBAT-1; D2) is either the carrier protein itself, forming a homooligomer, or an important component of a heterooligomeric structure (9, 12, 13). This protein (rBAT-1; D2) might be related to a brush border membrane function; previously, it was shown that small intestinal and proximal tubular brush border membranes seem to have a transport activity with a substrate specificity resembling b(+)P activity (3, 4, 14-16). Magagnini et al. (10) could demonstrate that rBAT-1-related mRNA species are also present in small intestinal epithelial cells and are capable of expressing b(+)P-related transport activity after oocyte injection. Northern blot analysis using a rBAT-1 cDNA probe has indicated the existence of two mRNA transcripts in various tissues, of 2.2 and 3.9 kb, respectively (9, 10). The 2.2-kb transcript corresponds in size to the cloned rBAT-1 cDNA and is about 2-3 times more abundant than the 3.9-kb transcript in rabbit kidney cortex (9, 10). The present work describes the isolation and characterization of a cDNA related to the 3.9-kb transcript. This long rBAT cDNA (rBAT-2) encodes a protein highly homologous to the protein related to the 2.2-kb transcript (rBAT-1); this conclusion is supported by sequence analysis, in vitro translation experiments, and expression of transport activity in oocytes. The difference between rBAT-1 and rBAT-2 seems to be primarily in the 3'-
untranslated region, i.e. most likely in the use of a different polyadenylation signal.

MATERIALS AND METHODS

X. laevis Oocytes and Transport Assay—All materials and methods concerning oocyte handling and amino acid transport have been described in detail (8, 9). Oocytes were injected with 2.5 ng of rBAT-1 or rBAT-2 cRNA, and 2–4 days after injection, transport of L-amino acids was measured at time periods showing a linear uptake rate (10 min). For transport of L-cystine, the uptake solution was supplemented with 10 mM diamide. Radiolabeled L-amino acids were obtained from Du Pont-New England Nuclear and used at an activity of 10–50 μCi/ml.

cDNA Cloning and Library Screening—All materials and methods used in cDNA library construction and screening, including cRNA transcription, have been described in detail previously (9, 17). In short, cDNA was synthesized from size-selected >2-kb rabbit renal cortex mRNA and then unidirectionally ligated into pBluescript SK+ (Stratagene). After plasmid transformation, 14,000 clones were plated and screened by in vitro transcribing the cDNAs and then injecting the cRNAs into oocytes and assaying for sodium-independent transport of L-amino acids. From initial pools of 1000 clones, the library was subdivided until a single clone (rBAT-2) was isolated, strongly stimulating sodium-independent transport of L-arginine, L-leucine, and L-cysteine, respectively.

cDNA Sequencing and Homology Analysis—rBAT-2 cDNA was sequenced using a double-stranded dideoxy chain termination technique (18) using α-35S-dCTP (New England Nuclear Radiochemicals) and a T7 DNA polymerase sequencing system (Pharmacia LKB Biotechnology Inc.). The cDNA was completely sequenced in both directions by using vector-derived primers and synthetic oligonucleotide primers. We have also generated a subclone of rBAT-2 (ligated into pBluescript SK+) by using HindIII-KpnI (base 2154 up to the 3′-end of rBAT-2).

mRNA Isolation and Northern Blots—RNA and mRNA samples were isolated according to previously published procedures (8). The cDNA probe for rBAT-1 was a 2160-bp BamHI-KpnI fragment lacking the 3′-end of the complete rBAT-1 cDNA, and for rBAT-2, a Smal-KpnI restriction fragment (from 3375 bp up to the 3′-end) was generated from the complete rBAT-2 cDNA. Both probes were labeled using α-32P-dCTP and the T7 Quick Prime Kit (Pharmacia). 10 μg of mRNA was electrophoresed on a 1% formaldehyde/agarose gel, transferred to GeneScreen membranes (Du Pont-New England Nuclear), and hybridized in a solution containing 50% formamide. After hybridization, the blots were washed four times quickly in 1 x standard saline citrate (SSC), 0.1% SDS at room temperature and then twice for 15 min at 45°C with 0.1 x SSC, 0.1% SDS.

In Vitro Translation—In vitro translation of rBAT-1 and rBAT-2 cRNAs was performed using a rabbit reticulocyte lysate system in the absence and presence of canine pancreatic microsomes (Promega). The reaction in the absence of microsomes was run in the presence of 0.5% (w/v) Triton X-100; otherwise the supplier’s protocol was followed (Promega). The reaction was stopped by addition of sample buffer (final concentration: 2% SDS, 20% glycerol, 1 mM EDTA, 120 mM Tris-HCl, 10 mM dithiothreitol, pH 6.8) and then directly loaded onto a 10% SDS-polyacrylamide gel. Electrophoresis and subsequent fluorography were performed according to standard protocols.

RESULTS

Similar to our previous procedure used to isolate the rBAT-1 cDNA (9), we have isolated an rBAT-2 cDNA by functional screening of a rabbit kidney cortex cDNA library (17) for expression of sodium-independent transport of L-arginine in X. laevis oocytes. A sib selection procedure resulted in the isolation of the rBAT-2 cDNA clone, which in contrast to the previously isolated rBAT-1 cDNA clone (9) has an insert 3.9 kb in length (data not shown) (see below). Injection of equal amounts of in vitro synthesized cRNA from both rBAT-2 and rBAT-1 cDNAs stimulated the uptake of L-arginine with approximately equal potency (about 15-fold; Fig. 1).

The insert of rBAT-2 cDNA was completely sequenced, and the sequence was compared at the nucleotide level (Fig. 2A) and amino acid level (Fig. 2B) with rBAT-1 (9). The rBAT-2 cDNA insert is 3934 bp in length, as compared with the shorter rBAT-1 cDNA, which is 2247 bp in length (9). A high homology (>99.9% identity) was observed between rBAT-1 and rBAT-2 cDNAs, within a common overlap of 2189 nucleotides, including the translation initiation site (double underlined), stop codon (marked by an asterisk), and first common polyadenylation signal (underlined). The predicted open reading frame for the rBAT-2-related protein encodes a protein of 677 amino acids, completely in accordance with rBAT-1, with only two amino acid differences, as deduced from the nucleotide sequence (Fig. 2B). Within the overlapping regions, there are seven nucleotide differences (five within the coding region), and the rBAT-2 cDNA has an additional 26 bp at the 5′-end (Fig. 2A). There is complete nucleotide identity in the first five nucleotides upstream of the start codon (ATG) in both clones, representing good consensus initiation sequences (19). The stop codons (TAG; 2032–2034 bp) are followed by an almost identical nucleotide sequence until reaching a first polyadenylation signal (AA-TAA; 2179–2184 bp) (20). In contrast to rBAT-1 cDNA, in which the poly(A) tail follows after a few bases, in rBAT-2 cDNA, this first polyadenylation signal is apparently not used, and a noncoding nucleotide sequence of ≈1.7 kb follows until reaching the poly(A) tail. This poly(A) tail is preceded by multiple short stretches (6 bases) rich in A, which might serve as polyadenylation signals.

The predicted amino acid sequence of rBAT-2 shows a lysine residue substituted for glutamic acid at position 41 and an alanine to proline substitution at position 213 when compared with rBAT-1 (Fig. 2B). Also, there is an identical secondary structure prediction for the proteins (Fig. 2B), with only one transmembrane domain according to the Kyte and Doolittle algorithm (amino acids 80–102; double underlined; Refs. 9 and 21) and an identical seven putative N-glycosylation sites (underlined) in rBAT-2 and rBAT-1 predicted proteins (9). In vitro reticulocyte translation of the rBAT-2 and rBAT-1 cRNAs resulted in several bands in both cases (Fig. 3A), the size of the major products being compatible.
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Fig. 2. A, comparison of the nucleotide sequences of rBAT-2 and rBAT-1. Nucleotides are numbered in the 5' → 3' direction, starting with the first nucleotide of the in-frame ATG codon (double underlined). The size of rBAT-2 is 3934 bp and contains a poly(A) tail of 63 bp, whereas rBAT-1 is 2247 bp in length with a poly(A) tail of 39 bp. The stop codon TAG lies at position 2032 bp (indicated by three asterisks) and is followed by a polyadenylation signal (AA-TAA) at position 2179 bp (underlined); if in rBAT-2 the nucleotide sequence immediately following the polyadenylation signal is shifted by two positions (TA in rBAT-1), there is an additional sequence identity for the next nine nucleotides. There are seven differences in base composition in the overlapping regions of rBAT-1 and rBAT-2 (boxed). At the 3'-end of rBAT-2, there is no canonical polyadenylation signal (19). Apparently, one of the A-rich stretches at the 3'-end of rBAT-2 is used as a polyadenylation signal (e.g. between bp 3781 and 3830). B, amino acid sequences of rBAT-2 and rBAT-1. The predicted open reading frames of both clones encode 677 amino acid residues. There are two amino acid differences between the two clones (boxed) at position 41 (lysine → glutamate) and position 215 (alanine → proline), respectively. The putative transmembrane domain (double underlined) is within amino acid residues 81 → 102. The seven potential N-glycosylation sites are underlined.

with the sizes predicted for the open reading frames (77,832 for rBAT-1 and 77,805 for rBAT-2). Addition of microsomes to the in vitro translation system resulted in a shift of these major translation products to higher molecular weights; en-doglycosidase H treatment shifted these proteins back to the size observed without addition of microsomes (data not shown). These observations further document that rBAT-1 and rBAT-2 code for almost identical proteins, most likely type II membrane glycoproteins (9, 22).

The available sequence information on rBAT-2 allowed us to generate a cDNA probe that should only recognize the 3.9-kb transcript in a Northern blot. As shown in Fig. 3B, a random primed cDNA probe derived from an ≈560-bp fragment at the 3'-end of rBAT-2 only recognizes the long transcript, which is in contrast to a cDNA probe corresponding to full-length rBAT-1, which identifies both short (2.2–2.3 kb) and long (3.7–3.9 kb) mRNA species (9, 10).

Sodium-independent transport of L-arginine (Fig. 1) could be either mediated by system y⁺-like or system b⁵⁺-like transport activity (see the Introduction). Injection of rBAT-2 cRNA into oocytes also led to the induction of L-cystine and L-leucine transport (Table I); L-cystine is not known to be transported by system y⁺-like transport activity (data not shown) (9, 10, 13). In Fig. 4, we show that sodium-independent transport of L-leucine and L-arginine is mediated by the same rBAT-2 induced transport activity. The induced L-arginine transport shows both self-inhibition (saturation) and inhibition by L-arginine. Similarly, the induced transport of L-leucine shows both self-inhibition (saturation) and inhibition by L-arginine. Furthermore, the rBAT-2-induced transport of L-arginine is inhibited by L-homoserine in the presence and absence of sodium (Fig. 5), which is typical for b⁵⁺-like activity (9, 10, 13). A system y⁺-like activity would show inhibition of L-arginine uptake by L-homoserine only in the presence of sodium (5, 7), as observed by the intrinsic uptake in water-injected oocytes (8–10, 13, 23). Thus, on the basis of this brief transport characterization, we conclude that rBAT-2-induced transport is identical to rBAT-1-related transport and most likely corresponds to a b⁵⁺-like activity (9, 10, 12).

J. Cunningham, personal communication.
DISCUSSION

We have isolated a cDNA clone of 3934 bp from a rabbit kidney cortex library, which most likely corresponds to the 3.7–3.9-kb mRNA transcript seen in Northern blots of various tissues, including rabbit kidney cortex mRNA. We could document that this cDNA (and this 3.7–3.9-kb mRNA species) is directly related to a cDNA of 2247 bp, which we earlier isolated and which in Northern blots recognizes a 2.2–2.3-kb transcript, as well as the above mentioned longer transcript (9).

The two cDNAs encode an almost identical protein of 677 amino acids in length. In the present study as well as in a previously documented one (9), we have shown that the expression of this protein in X. laevis oocytes leads to increased sodium-independent transport of dibasic amino acids, some neutral amino acids, and L-cystine via one single transport component (system b\textsuperscript{0}\textsuperscript{+}-like transport activity) (9, 10).

Previously, we named the short cDNA (2247 bp) rBAT-1 (9), corresponding to a protein involved in sodium-independent transport of amino acids in a variety of cell membranes (system b\textsuperscript{0}\textsuperscript{+}-like transport activity), including renal proximal tubular and small intestinal brush border membranes (see the Introduction and Refs. 1–4). Therefore, the longer (3934 bp) cDNA clone described in this paper was named rBAT-2.

We present experimental evidence to support our conclusion on the direct relationship of rBAT-2 to rBAT-1: sequence homology, identical in vitro translation products, and identical transport properties after the injection of the respective cRNAs into oocytes. The isolation of a second functional cDNA encoding a protein with the same apparent function in amino acid transport seems to be rather unusual; obviously, only clones with minor sequence alterations within the coding regions and compatible with function can be isolated using an expression cloning approach. Therefore, it is not surprising that we have only found five differences in the nucleotide composition, leading to two amino acid substitutions, within the encoding sequences of rBAT-2- and rBAT-1-related proteins. These sequence variations could arise by "errors" in cDNA synthesis or represent cDNAs synthesized from mRNA templates transcribed from different alleles of the rBAT gene. The major differences between rBAT-1 and rBAT-2 sequences are within the untranslated regions of the clones. At the 5'-end, the initiation codons are found within usual consensus sequences (19) and are preceded by only short nucleotide sequences. The 5'-ends may not be complete and might require primer extension analysis using the corresponding mRNAs as templates (17). In the 3'-untranslated region, rBAT-1 utilizes the first "canonical" polyadenylation consensus signal (20), which is in contrast to rBAT-2, where a noncoding sequence of roughly 1.7 kb is present in the clone. Interestingly, shifting the rBAT-2 nucleotide sequence by two positions (TA in rBAT-1) immediately after the polyadenylation signal results in an additional sequence identity of 9 bp between rBAT-1 and rBAT-2. The 3'-end of rBAT-2 contains numerous AT(U)-rich motifs, which apparently render such mRNA species highly unstable (20), suggesting that the rBAT-2-related mRNA might be degraded faster than the shorter rBAT-1-related mRNA species. This hypothetical difference in mRNA degradation/stability might offer an explanation for the apparent lower abundance of the longer transcript (rBAT-2) in mRNAs isolated from various organs and various species when compared with the shorter transcript (rBAT-1) (9); a similar explanation could be valid for the rat D2-related mRNA, which also shows a short (2.2 kb) and a long (4.4 kb) mRNA transcript (9, 10, 12). However, additional mechanisms responsible for different levels of...
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FIG. 3. A, in vitro translation of rBAT-2 and rBAT-1 cRNAs. The reactions were performed as described under “Materials and Methods,” in the presence (+) or absence (−) of microsomes. B, Northern blot analysis of rabbit kidney cortex mRNA using rBAT-1- and rBAT-2-specific probes. 10 μg of rabbit renal cortex mRNA was loaded per lane. rBAT-1 probe contains an almost complete rBAT-1 cDNA insert, whereas the rBAT-2-specific probe is a 560-bp sequence present at the 3’-end of rBAT-2 having no homology to the rBAT-1 transcript.

TABLE I
Expression of L-arginine, L-leucine, and L-cystine uptake by rBAT-2

<table>
<thead>
<tr>
<th>Uptake</th>
<th>H₂O</th>
<th>rBAT-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>0.63 ± 0.14</td>
<td>8.5 ± 0.46</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.22 ± 0.01</td>
<td>2.64 ± 0.18</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.06 ± 0.006</td>
<td>1.20 ± 0.12</td>
</tr>
</tbody>
</table>

expression of rBAT-1 and rBAT-2 in different tissues might have to be considered.

Using an expression cloning strategy, cDNAs with sequence alterations/truncations that are still compatible with biological function are selected. Therefore, the “selected” cDNA does not necessarily correspond (at least in length) to the major mRNA transcript present in the starting material (e.g. kidney cortex mRNA). As illustrated by the small differences in the coding region between rBAT-1 and rBAT-2, it also cannot be guaranteed that the coding sequence of an isolated cDNA indeed corresponds to the native major mRNA species; possible reasons for minor differences might be due to some heterogeneity in sequences in the available mRNA species (e.g. allelic variations), or the differences may be related to “cloning” artifacts.

Quite frequently, transcripts of different lengths are seen

FIG. 4. Inhibition by different amino acids of expressed transport of L-arginine (A) and L-leucine (B) in oocytes injected with rBAT-2 cRNA. Expressed transport is calculated by subtracting the intrinsic values (water-injected oocytes) from the induced transport activity (2.5 ng of rBAT-2 cRNA-injected oocytes). L-Arginine and L-leucine substrate concentrations were 50 μM, and inhibitors were added at 100-fold higher concentrations (5 mM). Uptake (10 min at 25 °C) was performed in the absence of sodium on the second day after injection. Data are shown as means ± S.D. for 6–10 oocytes for each condition. The experiment is representative of three similar experiments.

FIG. 5. Inhibition of L-arginine uptake by L-homoserine in water and rBAT-2 cRNA-injected oocytes. 50 nl of water or 50 nl of rBAT-2 cRNA (2.5 ng/oocyte) was injected into oocytes, and after 2 days, L-arginine (50 μM) uptake was measured (10 min) in 100 mM sodium chloride- or 100 mM choline chloride-containing media; L-homoserine was added at a concentration of 5 mM. Data are shown as means ± S.D. for 6–10 oocytes for each condition. The experiment is representative of three similar experiments.

in Northern blots, of which a single (mostly shorter) cDNA clone is “encoding” the function. They are usually “assigned” to the use of different polyadenylation signals. Within the field of expression cloning of membrane transport systems, we would like to describe a few examples. The human intes-
tinal Na-glucose cotransporter (SGLT-1) shows transcripts of 2.2, 2.6, and 4.8 kb, respectively, on Northern blots; the rabbit Na-glucose cotransporter shows only one transcript of 2.3 kb (24, 25). Similarly with rBAT-1/rBAT-2, the differences between the three separate human Na-glucose mRNA species have been suggested to be in the 3′-untranslated region. In the recent isolation of the osmotically regulated renal betaine transporter, a cDNA clone of 2938 bp was isolated by expression cloning, showing two signals of 2.4 and 3.0 kb, respectively, on the Northern blot (26). Also for the renal Na-myoinositol cotransporter, a cDNA of 2870 bp encoding full transport activity showed signals of much higher transcripts on Northern blots, with a band of 10.5 kb being the most frequent (27). Finally, the murine ecotropic retrovirus receptor, most likely encoding a protein involved in system Y-α amino acid transport activity, shows two transcripts of 7.9/9.0 and 7.0/7.5 kb on Northern blots, whereas the cDNA insert encoding the receptor/transport function is only about 2.4 kb in size (5, 6). These above examples seem to be in contrast to other transport systems, e.g., the different isoforms of Na/H exchangers, where only one transcript is observed for each of the isoforms (28–30). Unfortunately, in the above cases with multiple mRNA transcripts to be apparently related to one transport function, full sequence information and functional data are not available for two or more of the different mRNA transcripts. Thus, it cannot be predicted whether minor sequence alterations would still result in functional proteins and whether major differences between them are only in the 3′-untranslated region, e.g., by the use of a different polyadenylation signal. For such conclusions, complete sequencing of different functional cDNA clones would be required, as was done in the present study for rBAT-1 and rBAT-2.

It should be mentioned that a cDNA highly homologous to our rBAT-1/rBAT-2 has been isolated from rat kidney cortex (12, 13). As these cDNAs encode for a similar membrane protein with only one transmembrane region (type II membrane glycoprotein; Refs. 9, 12, and 22), their role in system b⁰⁰⁰-α like amino acid transport activity remains unclear. There are at least three different possibilities. (i) The rBAT-encoded protein is indeed the carrier protein and would most likely be required for function of a homomultimeric structure. (ii) The rBAT protein would represent an important subunit in a heteromultimeric carrier complex and would therefore associate with "silent" oocyte components to result in functional transport activity. (iii) The rBAT protein could be a specific factor required for specific activation (e.g., by an enzymatic reaction) of the observed transport activity (9, 12). As discussed in detail in the preceding papers, the rBAT protein shows some homologies to nonmembrane-bound α-amyloses and α-glucosidases (9, 12), however, without conservation of the specific amino acid residues required for hydrotropic activity. On the other hand, there is some interesting structural homology between the rBAT protein and the 4F2 surface antigen (heavy chain). Surprisingly, injection of 4F2-cRNA into oocytes showed expression of a system Y-α like amino acid transport activity (13). The above results led us to the proposal of an involvement of different proteins (e.g., the rBAT and 4F2 proteins) as specific subunits in specific amino acid transport systems (13). Clearly, this concept will require further examination for a better understanding.

In conclusion, we have isolated an rBAT-2 cDNA corresponding to the 3.9-kb transcript seen in Northern blots by expression cloning. rBAT-2 encodes a protein of 677 amino acids, different only in 2 amino acids from rBAT-1, as deduced by comparison with a previously isolated cDNA clone (rBAT-1) corresponding to a 2.2-kb transcript seen in Northern blots. Both rBAT-1- and rBAT-2-related proteins are involved in an identical manner in sodium-independent transport of dibasic amino acids, L-cystine, and some neutral L-amino acids (e.g., L-leucine and L-alanine) via a transport pathway closely resembling the specificity of system b⁰⁰⁰⁺.

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REFERENCES